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Firefly luciferase (Fluc) catalyzes a bioluminescent reaction using the substrates ATP and beetle luciferin in the presence of molecular oxygen (Fig. 1A). Because of its use of ATP and the simplicity of the single-enzyme system, firefly luciferase is widely used in numerous applications, notably those involving detection of living organisms, gene expression or amplification in both *in vivo* and *in vitro* systems.



Fluc has found intensive application in small animal *in vivo* bioluminescence imaging (BLI) in which the activity or state of labelled proteins, cells, tissues and organs may be localised

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and quantified sensitively and non-invasively. Different models and procedures for BLI are well described (Kung, 2005; Zinn *et al.*, 2008). For example, BLI is routinely applied to serially detect the burden of xenografted tumours in mice. Using more complex techniques, such as Fluc re-complementation, protein interactions such as chemokine receptor dimerisation (Luker *et al.*, 2008) have been imaged in small animals.

Recently, BLI has also been adapted to the detection of small molecules *in vivo* (Van de Bittner *et al.*, 2010). *D*-LH<sub>2</sub> is typically given intravenously or intraperitoneally to mice and has a broad biodistribution profile (Berger *et al.*, 2010). Cellular levels of Mg and ATP are sufficient to drive the reaction, though kinetics depends on substrate diffusion. Light emitted from labelled cells is detected using imagers which consist of CCD cameras in a dark box. This gives invaluable insight into the effects of experiments in the context of living organisms in real time.

The advantages of BLI over comparative techniques such as positron emission tomography (PET) include its simplicity, low cost, non-requirement for radiation and versatility. The other main optical imaging technique, fluorescence imaging (FLI), in which fluorescent small molecules or proteins are imaged in small animals, has lower signal to noise ratio than BLI due to the background signal in FLI from autofluorescence, quenching of signal due to endogenous tissue chromophores and also the requirement, and dependence on penetration, of an excitation light. Thus, BLI is approximately three orders of magnitude more sensitive than FLI and has a very large dynamic range (Wood, 1998).

All optical imaging techniques suffer from low resolution and from wavelength dependence of imaging due to photon scatter and signal attenuation by endogenous absorbing compounds. For example haemoglobin absorbs strongly below 590 nm. Therefore it is the red part of the spectra that is detected most efficiently (Caysa *et al.*, 2009).

Wild-type (WT) luciferase is highly thermolabile, inactivating and bathochromic shifting at even room temperature, and is sensitive to buffer conditions such as pH (Law *et al.*, 2006). The recombinant WT luciferase retains between 30 and 45% of activity at pH 7.0 relative to that at the optimal pH of 7.8 – 8.0, depending on whether flash heights or integrated light were measured (Law *et al.*, 2006). While many *in vitro* applications, such as those used to detect DNA amplification (Gandelman *et al.*, 2010) or ATP-assays (Strehler, 1968), take place at alkaline pH values of 8.0 or higher, *in vivo* applications such as medical or whole cell imaging must take place at neutral pH of 6.9 – 7.2, dependent on the exact cell type. At 37°C, WT Fluc shows a bathochromic shift and thus emits predominantly red light. Though light of red and longer wavelength does penetrate tissues more readily, red-shift luciferase bioluminescence is usually accompanied by a significant reduction in quantum yield and is therefore as such undesirable (Seliger and McElroy, 1959; Seliger and McElroy, 1960) as fluctuating levels of luciferase activity make quantitative studies problematic. Furthermore, for multispectral purposes (Mezzanotte *et al.*, 2011), any shift is undesirable. Therefore thermostable enzymes of different colours, which resist bathochromic shift are preferable.

The ideal Fluc would be highly thermostable, resist bathochromic shift, bright, have favourable kinetics (such as high substrate affinity) and have increased pH-tolerance. To address the issues of thermostability and bathochromic shift a number of recombinant mutant luciferases with increased thermal stability, giving brighter and more stable signals at elevated temperatures and resistant to bathochromic shift have been developed using

protein engineering (Hall *et al.*, 1999; Tisi *et al.*, 2002; Branchini *et al.*, 2009). Enhanced thermostability greatly improves the brightness achievable *in vivo*, and such enzymes are just recently finding application in animals (Law *et al.*, 2006; Baggett *et al.*, 2004; Mezzanotte *et al.*, 2011; Michelini *et al.*, 2008). A combination of higher thermal stability with increased pH-tolerance of Fluc is a very much desired and favourable feature for *in vivo* imaging which is likely to be useful in other applications (Foucault *et al.*, 2010).

Thermostability can be greatly improved by one amino acid change of FLuc and it has been observed that both changes in the enzyme core and on the protein surface can alter stability (Tisi *et al.*, 2002b). The substitutions A217I, L or V, identified by random mutagenesis increase the thermo- and pH-stability of *Luciola cruciata* and *L. lateralis* Flucs, and the equivalent substitution in *Photinus pyralis* (Ppy) Luc (A215L) also increases thermostability (Kajiyama and Nakano, 1993; Squirrell *et al.*, 1998). By random mutagenesis of Ppy Luc, and N-terminal surface loop-based substitutions, E354K or E354R have been identified to increase thermostability (White *et al.*, 1996). Combination of E354 with mutation D357 produced thermostable double mutants, of which E354I/ D357Y (x2 Fluc; Table 1) and E354R/ D357F were shown to be more stable than D357Y or E354K alone (Willey *et al.*, 2001).

Cumulative addition of such mutations further enhances thermostability. A typical example is a mutant containing T214C, I232A, F295L and E354K, named x4 Luc (Table 1) (Tisi *et al.*, 2002b). Non-conserved surface-exposed hydrophobic residues previously mutated to Ala (Tisi *et al.*, 2001; Prebble *et al.*, 2001) have also been substituted for polar ones (F14R, L35Q, V182K, I232K and F465R) to produce a mutant, named x5 Luc, displaying additively improved thermostability, solvent stability and pH-tolerance in terms of activity and resistance to red-shift; while retaining the same specific activity relative to WT luciferase (Law *et al.*, 2002; Law *et al.*, 2006). The most thermostable mutant luciferase, Ultra-Glo™ (UG) was created from *Photuris pennsylvanica* luciferase, and is commercially available for a number of assays (Hall *et al.*, 1999; Woodroffe *et al.*, 2008).

Majority of studies on improving thermostability, pH-tolerance and brightness of Fluc have been carried out using LH<sub>2</sub> until now. Emerging applications of luciferases in *in vivo* imaging of protease activity (Dragulescu-Andrasi *et al.*, 2009) require a different substrate - ALH<sub>2</sub>, one of the very few LH<sub>2</sub> analogues with which firefly luciferase also produces bioluminescence of relatively high intensity (White *et al.*, 1966). The substitution of the 6'-group extends the range of groups that can be conjugated to luciferin, for example to amino acids (Shinde *et al.*, 2006), peptides (Monsees *et al.*, 1995) and linear or bulky N-alkyl groups (Woodroffe *et al.*, 2008). Peptide-conjugated pro-luciferins allow the bioluminescent measurement of protease activity and in such applications ALH<sub>2</sub> (Monsees *et al.*, 1995) therefore the properties of different firefly luciferases and their mutants with ALH<sub>2</sub> may impact on the choice of enzymes applied.

The limited data on Ppy Fluc bioluminescence with ALH<sub>2</sub> as a substrate show that these properties are very different from those of LH<sub>2</sub>. The bioluminescence colour with ALH<sub>2</sub> has long been reported as pH-independent orange-red (max 605nm) (White *et al.*, 1966), Km for ALH<sub>2</sub> is approximately 26-times lower and Vmax is 10 times lower than that of LH<sub>2</sub> (Shinde *et al.*, 2006). There has been no further analysis of either red-shifted excited state emitter with

ALH<sub>2</sub> or its higher catalytic efficiency. From other studies it is known that there are luciferase isoforms from *Pyrophorus plagiophthalmus* that emit green light (*PpldGr*: 550 nm at pH 7.6) and yellow light (*PplvY*: 577 nm at pH 7.6) with ALH<sub>2</sub>. This indicates that red emission is not an intrinsic property of ALH<sub>2</sub>, but merely a consequence of enzymatic interactions and conformation of the active site (White *et al.*, 1966; Nakatsu *et al.*, 2006; Branchini *et al.*, 2001; Sandalova and Ugarova, 1999). Thus, it should be possible to engineer luciferase mutants with advantageous properties with ALH<sub>2</sub>, such as altered emission colour, higher activity and/or kinetics beneficial for *in vivo* imaging of protease activity.

In this paper we report on the construction and characterisation of a further improved x12 mutant based on the x5 mutant and seven additional mutations. Each of these mutations has previously been shown to confer slower rates of thermal inactivation (White *et al.*, 1996; Squirrell *et al.*, 1999; Tisi *et al.*, 2002). We compared the performance of x12 mutant with the WT *Ppy* and UG luciferases and demonstrated its pH tolerance and increased thermostability. A reversion of one of the mutations in the x12 resulted in a simplified mutant, termed x11 Fluc. Herein, we present properties of this mutant, which is highly thermostable, pH-tolerant, has high activity and catalytic efficiency with both LH<sub>2</sub> and ALH<sub>2</sub>, and presents a great potential for *in vivo* applications with both substrates.

## 2. Materials and methods

### 2.1 Materials

D-LH<sub>2</sub> potassium salt was obtained from Europa Bioproducts and D-ALH<sub>2</sub> from Marker Gene Technologies Inc. (Eugene, OR, USA). x2 Fluc was donated by Dr. Peter White (Dstl, Porton Down, Salisbury, UK) [White *et al.*, 2002; Willey *et al.*, 2001]. Ultra-Glo™ luciferase (UG) was purchased from Promega and all other chemicals were purchased from Melford Laboratories Ltd. or Sigma-Aldrich unless otherwise specified.

### 2.2 Construction of the x12 Fluc mutant and revertants

Seven mutations were introduced sequentially onto the x5 luciferase gene in pET16b-lucx5 (Law *et al.*, 2006) using the QuickChange™ Site Directed Mutagenesis (SDM) kit (Stratagene) according to the manufacturer's protocol. The primers used for the seven rounds of SDM are as follow:

5'-GCAGTTGCGCCCC**GTG**AACGAC-3' and 5'-GTCGTT**CAC**GGGCGCAACTGC-3' for A105V; 5'-CCCTATTTTCATT**CTG**GCCAAAAGCACTC-3' and 5'-GAGTGCTTTTGGC**CAG**GAATGAAAATAGGG-3' for F295L; 5'-GGCTACAT**ACT**GGAGACATAGC-3' and 5'-GCTATGTCTCC**AGT**ATGTAGCC-3' for S420T;

5'-CAAATCAAACCG**GGT**ACTGCGATTTTAAG-3' and 5'-CTTAAAATCGCAGT**ACCC**GGTTTGATTG-3' for D234G; 5'-CCGCATAGAT**TGT**GCCTGCGTCAGATTTC-3' and 5'-GAATCTGACGCAGGC**ACAT**CTATGCGG-3' for T214C;

5'-CACCC**CGC**GGGGATT**TAT**AAACCGGG-3' and 5'-CCCGGTTT**ATA**ATCCCC**GCG**GGGTG-3' (AvaI) for E354R and D357Y



Boldface type represents the mutated codon, underlined letters represent modified endonuclease site used to facilitate screening, and the endonuclease used for screening is shown in parentheses. *E. coli* BL21 (pLysS) (Edge Biosystems, Gaithersburg, MD, USA or XL2-Blue ultracompetent cells (Stratagene) were used as cloning hosts for the generation and selection of mutants from site-directed mutagenesis. Expression from colonies was induced by adsorbing colonies onto Hybond™-N nitrocellulose membranes (Amersham Biosciences Corp., Piscataway, NJ, USA) and transferring membranes onto fresh Luria Bertani (LB) agar plates containing 100 µg/ml carbenicillin and 1 mM IPTG and incubating for 3 hours at room temperature (RT). Bioluminescence was initiated by spraying membranes with 1 mM LH<sub>2</sub> or 500 µM ALH<sub>2</sub> in 0.1 M citrate buffer (pH 5) and colony screening was carried out by photographing emitted light with Nikon D70S camera (Nikon Corp., Tokyo, Japan). After seven rounds of SDM, mutations introduced were confirmed by sequencing of the entire luciferase gene using a facility provided by the Department of Genetics, University of Cambridge.

### 2.3 Expression and purification of ×12 Fluc and revertants

His<sub>10</sub>-tagged WT recombinant luciferase (WT) and mutants were expressed and purified according to the optimised protocol described in (Law *et al.*, 2006). Total protein concentrations were estimated by the method of Bradford (Bradford, 1976), using the Coomassie Blue protein assay reagent kit from Pierce according to the manufacturer's protocol, with BSA as the standard.

### 2.4 Luciferase activity assays, kinetic analysis, pH dependence of activity, thermal inactivation and bioluminescence spectra

Luciferase mutants were diluted from purified stock solutions into pre-chilled 0.1 M Tris/acetate; pH 7.8, 2 mM EDTA and 10 mM MgSO<sub>4</sub> (TEM) containing 2 mM DTT to obtain the required concentration, unless specified otherwise. Refer to caption accompanying each table or figure for method details. Bioluminescence spectra were captured using a Varian fluorometer (Palo Alto, Ca, USA). For measurements at differing pH values, TEM buffer at different pH values was used to dilute substrates and enzymes. Data were corrected for variant PMT sensitivity as previously described (Law *et al.*, 2006).

### 2.5 Mammalian cell culture, retrovirus production and transduction of cells

The genes encoding WT Fluc and x11 Fluc from pET16b constructs were cloned into mammalian retroviral expression vector SFG fused to Myc tags. These constructs were triple transfected into 293T cells, cultured in IMDM (Lonza, Basel, Switzerland) with 10% fetal calf serum (FCS) (Hyclone Labs Inc., Logan, UT, USA) and 1 % glutamax (Invitrogen Corp., Groningen, The Netherlands), along with plasmids encoding retroviral envelope and gagpol genes to produce retrovirus, which was used to transduce Raji cells. Transduced cells were sorted by flow cytometry using a Moflo-XDP instrument (Beckman Coulter, CA, USA) by anti-myc.FITC (Santa Cruz Biotechnology Inc., CA, USA) staining of the same mean fluorescence intensity and were cultured in RPMI 1640 (Lonza, Basel, Switzerland) with 10% FCS and 1 % glutamax in 5% CO<sub>2</sub>.

2.6 *In vivo* imaging

Three month old Beta2m-mice were tail vein injected with 1 x 10<sup>6</sup> Raji cells expressing WT or x11 Fluc. These were imaged using 30-60 s exposures in an IVIS 200 imager (Caliper, NJ, USA) at days 3 and 10 after anaesthesia with isofluorane and 15 min after intra-peritoneal (i.p.) injection of 200 µl of sterile D-luciferin (Regis Technologies, IL, USA).

3. Results and discussion

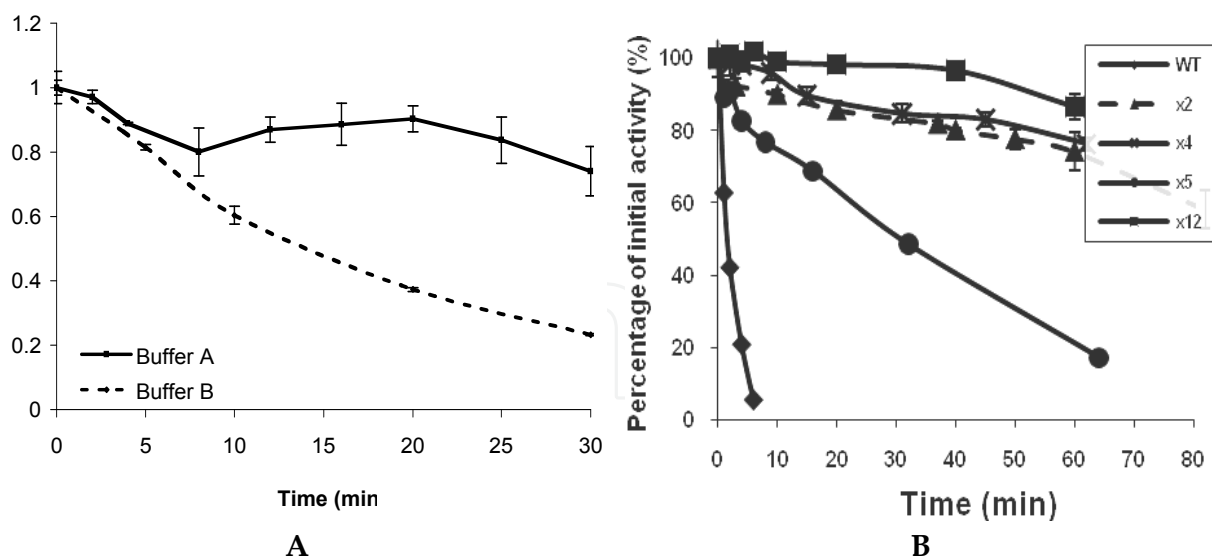
3.1 Construction, expression, purification and thermal inactivation of x12 Fluc and subset mutants

Seven additional mutations, previously shown to confer slower rates of thermal inactivation of Fluc, were sequentially added onto the x5 Fluc by SDM to create x12 Fluc, which was expressed in BL21(DE3)pLysS and purified to > 90 % homogeneity as previously described (Law *et al.*, 2006; White *et al.*, 1996; Tisi *et al.*, 2002; Squirrell *et al.*, 1999).

Mutation	Mutants					Location
	x12	x11	x5	x4	x2	
F14R	+	+	+			Surface
L35Q	+	+	+			Internal
A105V	+	+				Surface
V182K	+	+	+			Surface
T214A				+		Internal
T214C	+	+				Internal
I232A				+		Surface
I232K	+	+	+			Surface
D234G	+	+				Surface
F295L	+			+		Internal
E354R	+	+			+	Surface
E354K				+		Surface
D357Y	+	+			+	Surface
S420T	+	+				Surface
F465R	+	+	+			Surface

Table 1. Mutations and their positions in thermostable Fluc mutants

The loss of activity of x12 Fluc was measured at 55°C in two buffers, one of which allows direct comparison with the previously described x5 Fluc (Fig. 2A – buffer A) and the other, mimicking conditions used in BART (bioluminescent assay for monitoring nucleic acid amplification in real-time) (Fig. 2A – buffer B) (Tisi *et al.*, 2002) . x12 Fluc retained 80% of starting total activity after 30 min of treatment at 55°C, whereas x5 Fluc had < 1% activity remaining after 5 min at the same temperature (results not shown). When compared to previous thermostable Fluc mutants (Table 1) containing subsets of x12 Fluc mutations, x12 Fluc was the most resistant to thermal inactivation at 40°C (85-90% of initial activity after 1hr) (Fig. 2B), followed by x4 and x2 Fluc (both 75-80% after 1hr) and then x5 Fluc (20% after 1hr), which are all more stable than WT Fluc (fully inactivated within 10 min).



Thermal inactivation of x12 Fluc was determined at 55°C in two different conditions, namely 200 nM of x12 Fluc in Buffer A (50 mM phosphate buffer, pH 7.8, 10% glycerol (v/v), 2 mM DTT) and 86 nM of x12 enzyme in Buffer B (20 mM Tris.Cl, pH 8.8, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton X-100 (v/v), 5% trehalose (w/v), 0.5% BSA (w/v), 0.4 mg/ml PVP, 10 mM DTT). 30 µl aliquots of enzyme in the respective condition were incubated in water bath at 55°C for varying lengths of time up to 30 min. Enzyme activity was assayed by the injection of 100 µl of TEM, pH 7.8, 1 mM ATP, 200 µM LH<sub>2</sub> into wells containing 5 µl of enzyme and the measurement of flash height. PMT voltages used were 760 mV and 1000 mV for experiment in Buffer A and B respectively. Results shown are mean values ± S.E.M. for triplicate measurements (A). Flash-based activity with LH<sub>2</sub> was compared in aliquots of 0.5 µM enzyme incubated at set temperatures over time. Samples equilibrated to room temperature before dispensing 260 µl 70 µM LH<sub>2</sub> and 1 mM ATP solution in TEM buffer (pH 7.8) into 40 µl luciferase mutant (B).

Fig. 2. Thermal inactivation of x12 Fluc and subset mutants.

### 3.2 Effect of pH on x12 Fluc activity

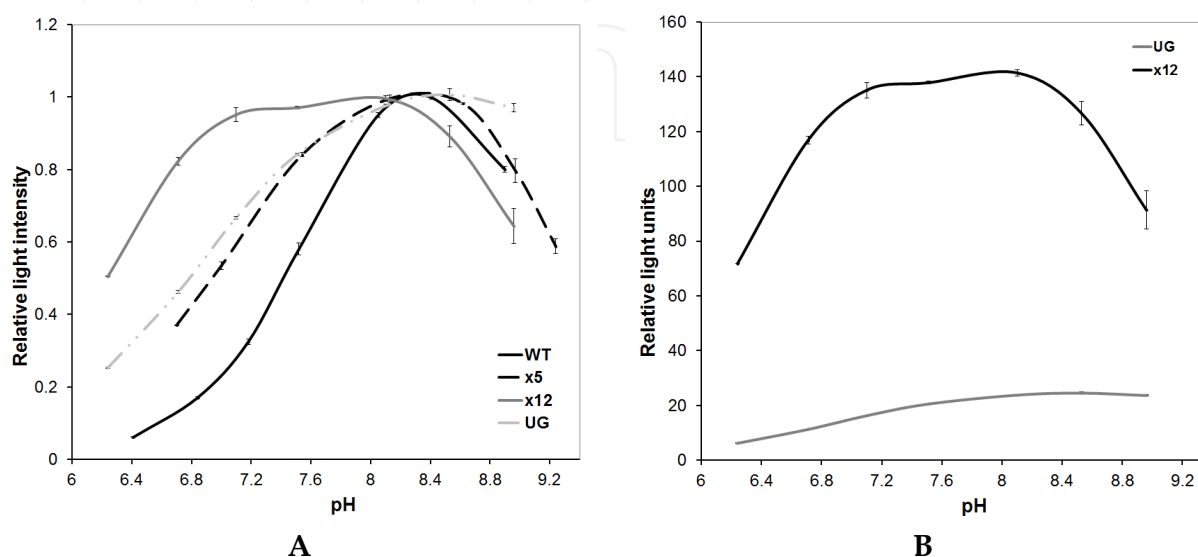
Detailed investigation on the pH-dependence of luciferase mutant activity revealed a significant further improvement in pH-tolerant profile from that of x5 Fluc (Law *et al.*, 2006). The normalised pH-dependence of activity was shown to facilitate comparison of activity across the range of pH values (Fig. 3A). The non-normalised results for x12 Fluc and UG emphasize the increase in activity for the x12 Fluc relative to UG (Fig. 3B). The high level of activity (≥ 80 % of maximum activity) exhibited by x12 Fluc across a range of physiologically relevant pH values (6.6 – 8.6) is likely to offer greater sensitivity and reliability when used in place of existing luciferase mutants in many applications, particularly those requiring a lower pH than the optimal for for Fluc or those that experience pH fluctuations such as whole cell or animal imaging (Frullano *et al.*, 2010).

### 3.3 Bioluminescence spectra and kinetic properties of WT Fluc, x12 Fluc and UG with LH<sub>2</sub> and ALH<sub>2</sub>

The bioluminescence spectrum of WT Fluc is known to undergo a classic red bathochromic shift with LH<sub>2</sub> at low pH, whereas x12 Fluc and UG maintained consistent yellow-green colours emission maximum of 557 nm and 560 nm respectively over the



investigated pH range of 6.2–8.8, (Table 2). Neither x12 nor UG showed any significant widening of the spectra at lower pH. Overall, bioluminescence spectra of x12 were even less pH-dependant than those of UG. Both thermostable mutants would be expected to retain their colour under physiological conditions, which may be pertinent to multispectral imaging. The tolerance of x12 Fluc to low pH may give it further advantage in terms of signal strength.



Luciferase activity (20  $\mu$ l at 0.42  $\mu$ M) was assayed by the manual mixing with 180  $\mu$ l of TEM, 1.11 mM ATP, 222  $\mu$ M LH<sub>2</sub>, 300  $\mu$ M CoA over a range of pH values between 6.0 and 9.5. Bioluminescence was integrated over 5 s using the luminometer at a PMT voltage of 550 mV: normalised to each luciferase enzyme total activity data (A) and non-normalised data (B). Measurements for UG were carried out using a PMT voltage of 700 mV to obtain good signal-to-noise ratio readings; data presented for the non-normalised curve have been corrected for the different PMT voltage used. The lag-time between initiation of the reaction and recording of light emission was  $\sim$  5s. Measurements at each pH were carried out in triplicate. Error bars represent one S.E.M. within triplicate measurements.

Fig. 3. Effect of pH on the activity of WT, UG, x5 and x12 Fluc.

Kinetic parameters including Michaelis-Menten constants ( $K_m$ ) and catalytic constant ( $K_{cat}$ ) were measured for the WT, x12 and UG luciferases with LH<sub>2</sub> and ALH<sub>2</sub>. The  $K_m$ s of x12 Fluc (6.8  $\mu$ M) and UG (3.7  $\mu$ M) for LH<sub>2</sub> were lower than that of WT Fluc (18  $\mu$ M) (Table 3), indicating an increase in affinity of the thermostable enzymes for LH<sub>2</sub> relative to the WT. Even stabilising mutations far from the active site can affect tertiary structure and lead to increased substrate affinities and altered enzyme kinetics (Squirrell *et al.*, 1999). Catalytic efficiency characterised by  $K_{cat}/K_m$  ratio was significantly compromised in both thermostable luciferases as compared to the WT, with UG being more thermostable but less catalytically efficient than x12. In terms of specific activity x12 Fluc retained only 15% of the specific activity of the WT, exceeding that of UG by four-fold.

For *in vivo* applications involving imaging of protease activity where ALH<sub>2</sub> is to be detected instead of LH<sub>2</sub>. Therefore, bioluminescence properties of these mutants with ALH<sub>2</sub> were tested. WT Fluc is known to produce pH independent orange-red emission with ALH<sub>2</sub> (White *et al.*, 1966) and here it was seen that WT Fluc, x12 Fluc and UG were red-shifted to

the emission maximum of 601 nm, 592 nm and 585 nm, respectively (Table 4). UG and x12 Fluc also had narrower half-bandwidth with ALH<sub>2</sub> than WT Fluc, which may indicate that more rigid active sites of UG and x12 Fluc confer better protection to the emitter than WT Fluc with ALH<sub>2</sub>.

Mutants	Bioluminescent spectra					
	$\lambda_{\text{max}}$ (nm)			Half-bandwidth (nm)		
	pH			pH		
	6.5	7.8	8.8	6.5	7.8	8.8
WT	592	559	558	91	66	66
x12	565	557	558	66	66	67
UG	562	560	559	73	68	70
x2	594	568	568	86	84	85
x4	560	558	561	69	66	70
x5	559	559	558	68	63	62

0.31 nmol of each mutant was added to 150  $\mu$ M LH<sub>2</sub> and 1 mM ATP in chilled 0.1 M TEM buffer, adjusted to a varying pH and spectra were measured after 30 s at ca. 25°C. Half-bandwidths are widths of spectra at half maximum intensity. Data were corrected for PMT spectral response. UG was assayed at pH 6.2 not 6.5. Standard error  $\pm$ 2nm.

Table 2. Bioluminescent spectra of the WT, Fluc mutants and UG with LH<sub>2</sub> at different pH

Mutants	Michaelis-Menten kinetic parameters			Specific activity	
	Km, $\mu$ M	Kcat x 10 <sup>8</sup> , RLU/s	Kcat/ Km x 10 <sup>13</sup> , RLU/ s x $\mu$ M	x 10 <sup>7</sup> , RLU/mg	% of WT at pH 7.8
WT	18 $\pm$ 1	1129 $\pm$ 215	668 $\pm$ 157	24.25 $\pm$ 0.14	100
x12	6.8 $\pm$ 0.2	50 $\pm$ 3	74 $\pm$ 2	3.56 $\pm$ 0.04	15
UG	3.7 $\pm$ 0.08	19 $\pm$ 2	53 $\pm$ 8	0.98 $\pm$ 0.02	4
x2	12 $\pm$ 2	1260 $\pm$ 181	1149 $\pm$ 110	11.37 $\pm$ 0.03	47
x4	28 $\pm$ 3	574 $\pm$ 84	201 $\pm$ 7	16.59 $\pm$ 1.53	68
x5	9.5 $\pm$ 1.5	1202 $\pm$ 142	1288 $\pm$ 51	21.33 $\pm$ 0.04	88

Kinetic parameters were derived from flash heights (Hanes, 1932) by varying LH<sub>2</sub> concentration in the range of 0.2 -500  $\mu$ M in the presence of saturating 1 mM ATP (see Materials and Methods). Specific activity was determined in the presence of 20.1 pmol enzyme by integrating light in 20 ms pulses over 12 s. PMT voltage 550 mV.

Table 3. Kinetic parameters of WT, Fluc mutants and UG with LH<sub>2</sub>

In terms of kinetic parameters significant differences were observed in Km and Kcat values for LH<sub>2</sub> and ALH<sub>2</sub> between the WT and thermostable luciferases. In the WT both Km and Kcat for ALH<sub>2</sub> were significantly lower than for LH<sub>2</sub>, suggesting that WT Fluc has higher affinity for ALH<sub>2</sub> than for LH<sub>2</sub> (Shinde *et al.*, 2006). In thermostable x12 Fluc both Km and Kcat for ALH<sub>2</sub> were very close to those of LH<sub>2</sub>, while in UG Km for ALH<sub>2</sub> was higher and Kcat was lower than for LH<sub>2</sub>. The mutations that invoke thermostability increase the affinity of Fluc for LH<sub>2</sub>, but reduce it for ALH<sub>2</sub>. WT Fluc had nearly 10-fold higher catalytic

efficiency (Kcat/Km) with LH<sub>2</sub> and ALH<sub>2</sub> than x12 Fluc. x12 demonstrated comparable Kcat/Km ratio for both substrates, favouring its use with ALH<sub>2</sub> while UG had a similar catalytic efficiency to x12 Fluc for LH<sub>2</sub>, but 23-times lower Kcat/Km for ALH<sub>2</sub> . Overall, x12 Fluc seems to be a mutant of choice to be used with ALH<sub>2</sub>.

Mutants	Bioluminescent spectra					
	λmax (nm)			Half-bandwidth (nm)		
	pH			pH		
	6.5	7.8	8.8	6.5	7.8	8.8
WT	599	601	601	74	79	79
x12	596	592	591	72	72	71
UG	588	585	584	71	72	71
x2	596	596	595	76	76	74
x4	596	597	593	74	74	74
x5	599	600	599	74	76	73

0.31 nmol of each mutant was added to 100 μM ALH<sub>2</sub> and 3 mM ATP in chilled 0.1 M TEM buffer, adjusted to a varying pH and spectra were measured after 30 s at ca. 25°C. Half bandwidths are widths of spectra at half maximum intensity. Data were corrected for PMT spectral response. UG was assayed at pH 6.2 not 6.5. Standard error ±2nm.

Table 4. Bioluminescent spectra of the WT, Fluc mutants and UG with ALH<sub>2</sub> at different pH

WT Fluc appears to provide stringent interactions dictating reaction kinetics with ALH<sub>2</sub>, but not those that govern the emitting species. This may also be indicated by larger half-bandwidths of bioluminescence spectra with ALH<sub>2</sub> than with LH<sub>2</sub>. Low K<sub>m</sub> values were proposed to correlate with more blue-shifted emission (Kutuzova *et al.*, 1997), but this is not always true and it is possible for K<sub>m</sub> to be more linked to bioluminescence spectra half-bandwidth, i.e. the extent of vibrational freedom of oxyluciferin (Viviani *et al.*, 2001). However, here, WT Fluc has wider spectral half-bandwidth with ALH<sub>2</sub> as compared to LH<sub>2</sub>, but lower K<sub>m</sub>. WT Fluc had only 10% of the specific activity with ALH<sub>2</sub> as compared to LH<sub>2</sub> at pH 7.8 (Table 5). On the contrary, x12 Fluc’s activity with ALH<sub>2</sub> (6%) is only 2.5-fold lower than that with LH<sub>2</sub> (15%), while UG had as low as 4% with both substrates. Reasons for lower V<sub>m</sub> and red-shifted emission of WT Fluc with ALH<sub>2</sub> have been postulated (Shinde *et al.*, 2006; McCapra and Perring, 1985; Wada *et al.*, 2007).

3.4 Properties of thermostable mutants containing subsets of x12 Fluc mutations

Elimination of bathochromic shift at low pH, increase in thermostability and pH-tolerance were associated with the significant reduction in specific activity and changes in the essential kinetic parameters. In an attempt to identify mutations responsible for the undesirable associated changes and to improve the understanding of kinetics and activity differences between WT Fluc and x12 Fluc with LH<sub>2</sub> and ALH<sub>2</sub> a number of thermostable enzymes with subsets of x12 Fluc mutations were investigated (Table 1). These were x2 Fluc (E354R/ D357Y) (Baggett *et al.*, 2004), x4 Fluc (T214A/ I232A/ F295L/ E354K) (Tisi *et al.*, 2002) and x5 Fluc (F14R/ L35Q/ V182K/ I232K/ F465R) (Law *et al.*, 2006). Among the subset mutants only x2 showed bathochromic shift with LH<sub>2</sub> at low pH, similar to that

observed in the WT (Table 2). x4 and x5 Flucs resisted red-shift at low pH in line with x12. With ALH<sub>2</sub> none of the subset mutants showed any significant shift in the bioluminescence across the investigated pH range, which was similar to the behaviour of x12, WT and UG with this substrate (Table 4).

Mutants	Michaelis-Menten kinetic parameters			Specific activity	
	Km, $\mu$ M	Kcat $\times 10^8$ , RLU/s	Kcat/ Km $\times 10^{13}$ , RLU/ s $\times \mu$ M	$\times 10^7$ , RLU/mg	% of WT with LH <sub>2</sub> at pH 7.8
WT	2.4 $\pm$ 0.4	174 $\pm$ 13	849 $\pm$ 158	2.06 $\pm$ 0.01	8
x12	6.5 $\pm$ 0.2	38 $\pm$ 4	59 $\pm$ 9	1.46 $\pm$ 0.01	6
UG	8.1 $\pm$ 2.19	1.4 $\pm$ 0.04	2.3 $\pm$ 0.7	0.60 $\pm$ 0.01	4
x2	2.3 $\pm$ 0.1	226 $\pm$ 24	956 $\pm$ 68	1.99 $\pm$ 0.03	8
x4	9 $\pm$ 1	144 $\pm$ 14	166 $\pm$ 14	2.51 $\pm$ 0.03	10
x5	1.6 $\pm$ 0.1	215 $\pm$ 32	1343 $\pm$ 184	2.07 $\pm$ 0.01	9

Kinetic parameters were derived from flash heights (Hanes, 1932) by varying ALH<sub>2</sub> concentration in the range of 0.1-600  $\mu$ M used with 3mM ATP to saturate (see Materials and Methods). Specific activity was determined in the presence of 20.1 pmol enzyme by integrating light in 20 ms pulses over 12 s. PMT voltage 550 mV.

Table 5. Kinetic parameters of WT, Fluc mutants and UG with ALH<sub>2</sub>

In terms of kinetic parameters, if compared to the WT, the effect of mutations in x4 on Km and Kcat with LH<sub>2</sub> differed significantly from mutations in both x2 and x5 subsets (Table 3). Mutations in x4 increased the Km, while in two other subsets decreased it, and reduced the Kcat, while in the other subsets hardly any change was observed. These results correlated well with the previously reported in literature (Law *et al.*, 2006; Tisi *et al.*, 2002; Prebble *et al.*, 2001; Branchini *et al.*, 2007). Similar trends were observed in the kinetic parameters for ALH<sub>2</sub> (Table 5). Mutations in x4 significantly increased its Km for this substrate (as in x12), while two other mutants slightly decreased it. Kinetics of the x4 mutant with ALH<sub>2</sub> exhibited slower rise and slower decay, reminiscent of the kinetics of x12, whereas x2 and x5 displayed a sharp flash that strongly resembled the WT.

The differences in kinetic parameters and flash kinetics suggested that an undesirable effect of mutations was common to x4 and x12 mutants, but not to x2 or x5, on catalysis with both LH<sub>2</sub> and ALH<sub>2</sub>. It was hypothesised that reversion of buried x12 Fluc mutations T214C and F295L might enhance its catalytic properties.

3.5 Properties of T214 and F295 revertant mutants of x12 Fluc

x12 Fluc mutations T214C and F295L were individually reverted and their bioluminescent properties, catalytic parameters and thermal stability investigated. Bioluminescence spectra of both revertants were measured and found to match those of x12 Fluc with both substrates (Table 6). F295 revertant showed no bathochromic shift with either substrate and retain the pH-tolerance previously observed in x12 Fluc. T214 revertant had a flash kinetics similar to

x12 Fluc with both substrates, but F295 had a faster and brighter flash with LH<sub>2</sub>, more resembling that of the WT (not shown).

Mutants	Substrate	Bioluminescent spectra					
		λmax (nm)			Half-bandwidth (nm)		
		pH			pH		
		6.5	7.8	8.8	6.5	7.8	8.8
x12	LH <sub>2</sub>	565	557	558	66	66	67
	ALH <sub>2</sub>	596	592	591	72	72	71
T214	LH <sub>2</sub>	-	560	-	-	66	-
	ALH <sub>2</sub>	-	591	-	-	72	-
F295	LH <sub>2</sub>	563	559	560	71	66	70
	ALH <sub>2</sub>	593	592	591	70	72	73

Details are as in Table 2 and 4.

Table 6. Bioluminescent spectra of x12 Fluc and its revertants at different pH with LH<sub>2</sub> and ALH<sub>2</sub>

T214 revertant exhibited Kms and Kcats similar to x12 Fluc for LH<sub>2</sub> and its Kcat/Km ratio was not significantly different from that of x12 Fluc (Table 7). The Km of T214 for ALH<sub>2</sub> was slightly lower than x12 Fluc and Kcat elevated, resulting in the higher Kcat/Km ratio. T214 revertant had similar specific activities to x12 Fluc with both substrates. However, the Km of F295 revertant for both LH<sub>2</sub> and ALH<sub>2</sub> was lower and Kcat was higher than that of x12 Fluc resulting in a much higher Kcat/Km ratio. Specific activity of F295 revertant with both substrates was two-fold brighter than that of x12 Fluc. As a result of these findings F295 revertant afforded higher sensitivity to the flash-based detection of LH<sub>2</sub> and ALH<sub>2</sub> than x12 Fluc. It is possible to conclude that in x12 Fluc residue F295 contributes much more to catalysis than T214 and its reverse-mutation may significantly improve the performance of x12 Fluc providing it has no negative effect on the thermostability.

Mutants	Substrate	Michaelis-Menten kinetic			Specific activity	
		Km, μM	Kcat x 10 <sup>8</sup> , RLU/s	Kcat/ Km x 10 <sup>13</sup> , RLU/ s x μM	x 10 <sup>7</sup> , RLU/mg	% of x12 with LH <sub>2</sub> at pH 7.8
x12	LH <sub>2</sub>	6.8+0.2	50+3	74+2	3.56+0.04	100
	ALH <sub>2</sub>	6.5+0.2	38+4	59+9	1.46+0.01	40
T214	LH <sub>2</sub>	6.2+0.5	49+3	83+10	3.22+0.03	79
	ALH <sub>2</sub>	4.9+0.5	66+2	138+8	2.37+0.05	58
F295	LH <sub>2</sub>	3.7+0.2	133+6	366+22	8.03+0.09	197
	ALH <sub>2</sub>	3.6+0.2	82+2	231+9	3.23+0.02	79

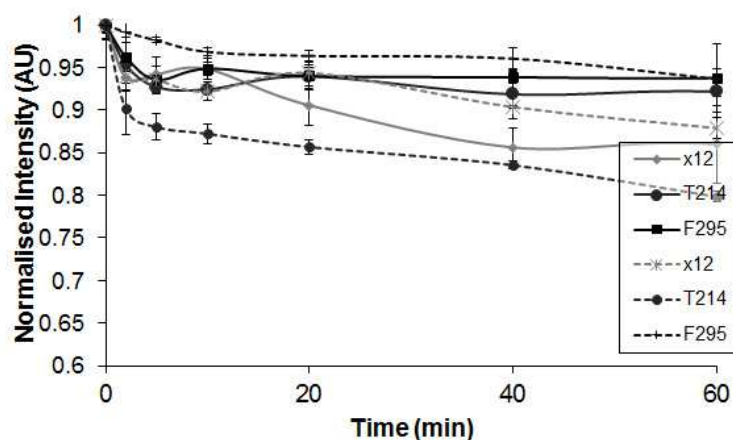
Details are as in Table 3 and 5. Specific activity was measured at pH 7.7 with LH<sub>2</sub> and at pH 8.2 with ALH<sub>2</sub>.

Table 7. Kinetic parameters of x12 Fluc revertants with LH<sub>2</sub> and ALH<sub>2</sub>

Although T214C and F295L are thermostabilising mutations (Law *et al.*, 2006; Tisi *et al.*, 2002), revertants displayed resistance to thermal inactivation similar to that of x12 Fluc at 40°C and 50°C (Fig. 4). Therefore, the mutation F295L is not essential for practically useful



thermostability properties of x12 Fluc, but in the framework of this enzyme contributes to reduced activity and catalytic efficiency with both LH<sub>2</sub> and ALH<sub>2</sub>. F295 revertant was hereafter named x11 Fluc. Cumulative addition of multiple phenotype-inducing mutations may enhance desired mutant properties, but some additions can clash and unduly disrupt properties.



Flash-based activity with LH<sub>2</sub> was compared in aliquots of 0.5  $\mu$ M enzyme incubated at set temperatures over time. Samples were equilibrated to RT before dispensing 260  $\mu$ l of 70  $\mu$ M LH<sub>2</sub> and 1 mM ATP solution in TEM buffer (pH 7.8) onto 40  $\mu$ l luciferase mutants. Solid lines: 40°C, dashed lines: 50°C.

Fig. 4. Thermal inactivation of x12 Fluc and revertants at 40°C and 50°C.

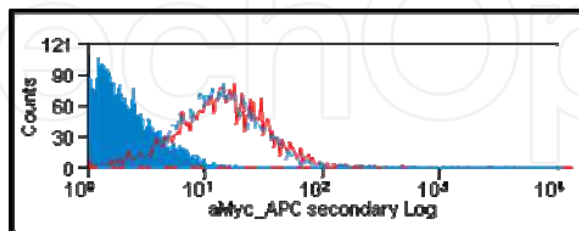
### 3.6 *In vivo* imaging

Human retrovirus encoding *Ppy* Fluc or x11 Fluc genes bearing a myc tag was used to transduce Raji cells, which were sorted to the same expression levels (Fig. 5A) by flow cytometric sorting for myc tag staining and cultured at 37°C. As an example of *in vivo* imaging using x11 Fluc, one million Raji cells expressing similar levels of WT or x11 Flucs were injected into the tail veins of immunocompromised Beta2m-mice to induce systemic lymphoma (Chao *et al.*, 2011) and imaged after i.p. administration of LH<sub>2</sub>. Images revealed light signals predominantly from brain, spine and hips. x11 Fluc appeared very bright *in vivo* (Fig. 5B) because of its high thermostability and pH tolerance along with favourable kinetic parameters demonstrated in characterisation. It is expected to perform equally well under the changing physiological pH conditions and in combination with aminoluciferin used for imaging of protease assays *in vivo*. Further work to codon-optimize and test this mutant in mammalian cells is underway and will be published shortly.

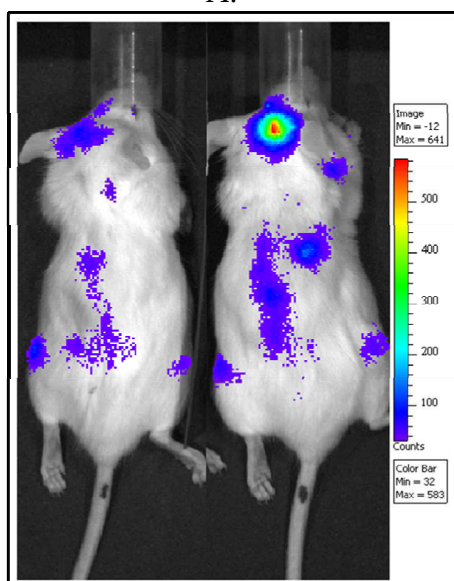
## 4. Conclusion

In the present study, we describe the construction and characterisation of the firefly luciferase mutant x12 Fluc based on the mutations previously identified as increasing thermostability of the enzyme. Detailed characterization of its bioluminescent and biochemical properties revealed that it is the only luciferase mutant reported to exhibit  $\geq 80\%$  of total activity across a wide pH range of 6.6 - 8.8 covering physiologically

significant pH at the lower end. Additionally, it possesses sufficient thermal stability to be applicable to assays that require temperatures lower than 50°C or in assays involving short lengths of higher temperature exposure. This mutant could be beneficial for *in vivo* imaging with luciferin, particularly in assays that experience pH fluctuations, and for bioluminescence protease assays or *in vivo* protease imaging, in which the sensitivity of detection may be dependent on the sensitivity of ALH<sub>2</sub> detection. The latter assays could benefit further from increased brightness.



A.



B.

FACS histogram showing expression of WT Fluc and thermostable mutants in Raji cells. Cells obtained by fluorescence activated cell sorting were expanded *in vitro* and expression of transgenes was analysed using a Cyan flow cytometer using anti-myc.FITC staining. Non-transduced cells (blue line – filled) were compared to WT Fluc (red line) and x11 Fluc (blue line – dashed) (A). *In vivo* imaging of systemic lymphoma expressing WT Fluc (left) and x11 mutant (right). Mice with lymphomas expressing either WT Fluc or x11 Fluc were imaged in three groups of three mice (B).

Fig. 5. Expression and example of *in vivo* imaging of Flucs in mammalian cells.

The brightness of x12 Fluc was improved by identifying the mutation at the 295 position as having the major negative impact on the bioluminescent characteristics, reverting it and creating x11 Fluc, which possessed all the desirable properties. x11 Fluc has remarkably high activity and catalytic efficiency with LH<sub>2</sub> and ALH<sub>2</sub>, coupled to a high resistance to thermal inactivation and pH-tolerance. Its highly advantageous properties in terms of stability and brightness in mammalian cells have been demonstrated using systemic lymphoma expressing the mutant in mice as an example, as there was no normalisation for engraftment and no statistical difference between WT and x11 in this small sample set.

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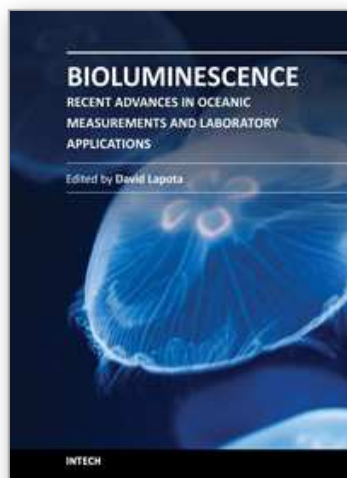


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We now find ourselves utilizing luciferase - luciferin proteins, ATP, genes and the whole complex of these interactions to observe and follow the progress or inhibition of tumors in animal models by measuring bioluminescence intensity, spatially and temporally using highly sophisticated camera systems. This book describes applications in preclinical oncology research by bioluminescence imaging (BLI) with a variety of applications. Chapters describe current methodologies for rapid detection of contaminants using the Milliflex system, and the use of bioluminescence resonance energy transfer (BRET) technology for monitoring physical interactions between proteins in living cells. Others are using bioluminescent proteins for high sensitive optical reporters imaging in living animals, developing pH-tolerant luciferase for brighter in vivo imaging, and oscillation characteristics in bacterial bioluminescence. The book also contains descriptions of the long-term seasonal characteristics of oceanic bioluminescence and the responsible planktonic species producing bioluminescence. Such studies are few and rare.

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